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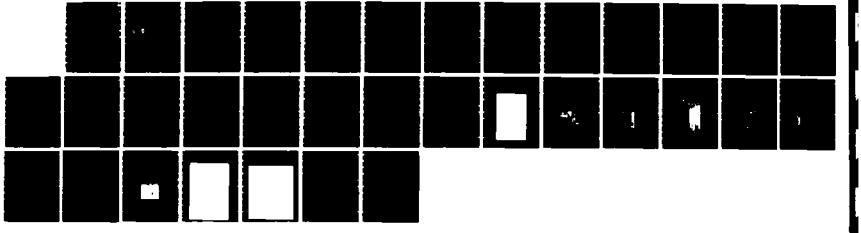

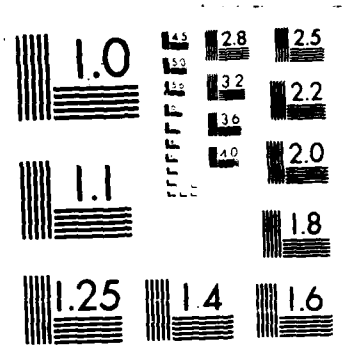

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TITLE

Development of a DNA-Based Method for Distinguishing the Malaria Vectors, Anopheles gambiae from Anopheles arabiensis

TYPE OF REPORT

Annual Report

AUTHOR

Victoria Finnerty, Ph.D.

DATE

June 1986

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Ft. Detrick, Frederick, MD 21701-5012

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Emory University
Atlanta, GA 30322

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The Anopheles gambiae complex includes six morphologically identical species, two of which (A. gambiae and A. arabiensis) are the primary African malaria vectors today. Since two or more of the species are commonly sympatric, epidemiological studies to determine the involvement of each in malaria transmission have been difficult. This report describes our efforts to identify a DNA segment from A. gambiae which when used as a probe on Southern blots containing both A. gambiae and A. arabiensis DNA will reveal a restriction fragment length polymorphism. Thus far we have successfully cloned an rDNA gene from an A. gambiae genomic library. From this we have obtained a .5kb EcoRI-SalI subcloned fragment which shows hybridization to about a 6kb EcoRI fragment in A. arabiensis and to a 1.5kb EcoRI fragment in A. gambiae. These diagnostic bands are consistently seen on blots of whole genomic DNA. The method can also be used on dried single mosquitoes or mosquito abdomens although the DNA extraction method currently used does not always yield

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SUMMARY

The Anopheles gambiae complex includes six morphologically identical species, two of which (A. gambiae and A. arabiensis) are the primary African malaria vectors today. Since two or more of the species are commonly sympatric, epidemiological studies to determine the involvement of each in malaria transmission have been difficult. This report describes our efforts to identify a DNA segment from A. gambiae which when used as a probe on Southern blots containing both A. gambiae and A. arabiensis DNA will reveal a restriction fragment length polymorphism. Thus far we have successfully cloned an rDNA gene from an A. gambiae genomic library. From this we have obtained a .5kb EcoRI-SalI subcloned fragment which shows hybridization to about a 6kb EcoRI fragment in A. arabiensis and to a 1.5kb EcoRI fragment in A. gambiae. These diagnostic bands are consistently seen on blots of whole genomic DNA. The method can also be used on dried single mosquitoes or mosquito abdomens although the DNA extraction method currently used does not always yield sufficient DNA for the test.

FOREWORD

Studies with Recombinant DNA: The investigator has abided by the National Institutes of Health Guidelines for Researach Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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ANNUAL REPORT

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2. Principal Investigator: Victoria Finnerty, Ph.D.

3. Date of Report: 15 June 1986

4: Address: Dept. of Biology, Emory University, Atlanta, GA 30322

5. Project Title: Development of a DNA-based method of distinguishing the malaria vectors A. gambiae from A. arabiensis.

6. Statement of Problem Under Study:

A. gambiae and A. arabiensis, the two most prominent African malaria vectors are morphologically indistinguishable (1,2). However, biological evidence suggests that these two sympatric species may not be equally involved in malaria transmission in those areas where they co-exist (3-4). The resolution of this important epidemiological question requires a reliable means for species identification of individual field specimens. Moreover, such specimens must also be examined for the presence of the malaria sporozoite. At present, the only useful means for species identification of adults is examination of the nurse cell polytene chromosomes (5). Alternative procedures based on electrophoretic enzyme variation or cuticular hydrocarbon profiles determined by HPLC (6) are not reliable. It is clear that none of the above-mentioned procedures are practical epidemiological tools for field specimens. On the other hand, several types of reliable immunological procedures for detecting sporozoites in dried field material have recently been developed (7-11). Therefore, a very useful addition to these studies would be a means of reliably identifying the species of single dried mosquitoes. This report will discuss our current efforts to develop such an assay.

7. Background:

Many of the major malaria vectors are members of species complexes, for instance, A. culicifacies (12), A. leucosphyrus (13), and the A. farauti sibling series (14). In these complexes, as well as in the A. gambiae complex, reliable species identification of individuals is currently tedious and difficult. Since malaria continues to represent a major world health problem, epidemiological studies with these species is crucial.

Our studies are focused on two sympatric species, A. gambiae and A. arabiensis. The original proposal hypothesized that the genomic DNA of these two species currently differs in ways that would permit reliable species identification. In particular, we sought to develop a species differentiating assay based upon restriction fragment length polymorphism as detected by either heterologous or species-specific probes.

8. Rationale:

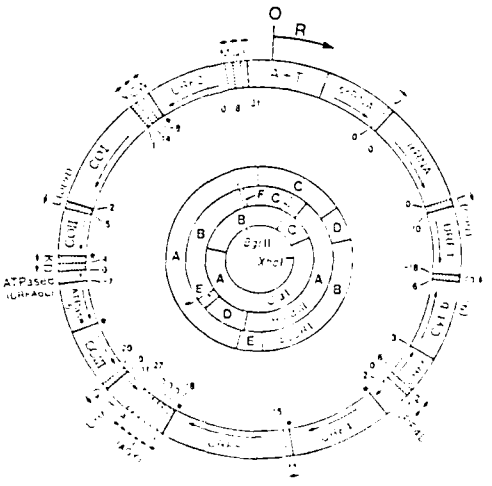
A substantial body of evidence argues that RFLP exist between members of related species (15,16,17). Given that we expected to find such differences between A.

gambiae and A. arabiensis, it seemed that a DNA-based assay would be an ideal epidemiological tool. The major advantages of a DNA-based assay should be the ability to use dried material and the sensitivity of Southern analysis.

9. Experiments and Results:

(A) Heterologous Probes. Our proposal suggested an initial survey of heterologous probes which are known to be highly conserved throughout various phyla (18,19). One choice was the Drosophila actin sequence (20) because in addition to its highly conserved sequence, it is a member of a multigene family, thus giving us more copies per genome for detection of single mosquito patterns by Southern analysis. However, in our preliminary studies hybridization with the Drosophila actin probe required such low stringency conditions that the signal to noise ratio made the blots impossible to interpret. We quickly decided to examine appropriate mosquito sequences to use as probes for distinguishing RFLPs. Moreover, since our long term goals include isolation of species-specific mosquito sequences, we did not examine any other heterologous probes.

(B) Mosquito Mitochondrial DNA Probes. Studies of mitochondrial RFLPs have been widely used to construct the phylogeny of closely related species. We therefore decided to utilize this approach for A. gambiae and A. arabiensis. We obtained a clone pDyHB from David Wolstenhome, bearing a 4.8kb HindIII fragment from Drosophila yakuba which includes the cytochrome oxidase I and II genes. Since these genes are highly conserved, we sought to use this fragment to identify a homologous sequence in A. gambiae. When pDyHB (shown below in red) was used to probe A. gambiae DNA restricted with various enzymes, Southern analysis showed a small number of hybridizing bands.



The combined molecular size of these bands was in no case greater than that (14-17kb) expected for the mitochondrial genome. One of the hybridizing bands is a 5.5kb EcoRI fragment which is a convenient size for cloning into the lambda-gt10 vector. We therefore electroeluted 5-6kb DNA from a preparative EcoRI digest, ligated the fragments with lambda-gt10, and packaged the phage using the commercially available "Gigapack." The resulting minilibrary was screened using lowered stringency (hybridization and washing at 42°C) with the pDyHB probe.

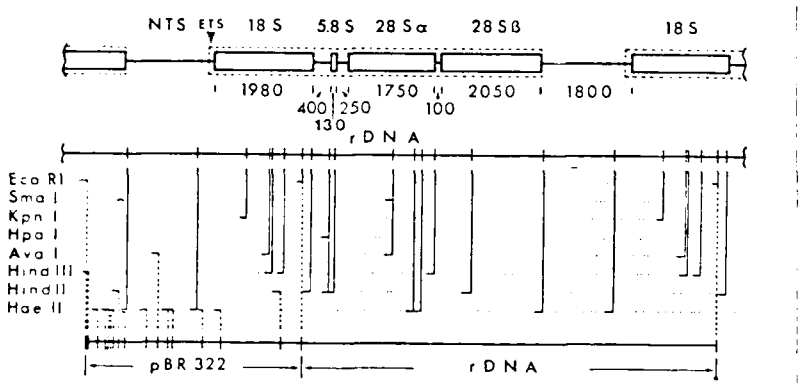
One of the selected clones, MR7, had a 5.5kb insert which was used for further study. Of the several enzymes tried, the most interesting differences between gambiae and arabiensis were seen with PstI digests probed with MR7, an example of which is shown as figure 1. In addition to the interspecies difference, there is also a suggestion that this probe may be useful for diagnosing various geographical isolates of a given species, such as those listed in Table 1. We did not investigate this interesting possibility any further.

One question concerning MR7 is whether it actually represents mitochondrial DNA. One possibility would be that it is actually a nuclear DNA sequence containing a tRNA also present (see hatched areas above) in mitochondria. Since tRNA sequences tend to be highly conserved, the D. yakuba probe could have selected either nuclear or mitochondrial sequence. We found that the fragment of pDyHC (shown above in green) also hybridizes to MR7, which indicates that MR7 most likely is a mitochondrial sequence. However, we have not characterized MR7 any further and therefore we cannot make definite statements concerning its origin. Regardless of its origin, MR7 is formally equivalent to a mitochondrial probe since it hybridizes to mitochondrial DNA.

In summary, we found interesting differences between A. gambiae and A. arabiensis using MR7, but even the PstI pattern differences (shown in figure 1) would be difficult to discern with confidence if one were using single mosquitoes. We might have then cloned other portions of the mosquito mitochondrial genome to see if a more useful probe could be found, had we not already obtained interesting preliminary data from mosquito rDNA clones. Thus we began to examine rDNA probes which might be more useful as potential diagnostic tools.

(C) Mosquito rDNA Probes. The rDNA genes of most eukaryotic organisms contain non-transcribed spacer regions (NTS) which have been shown to diverge quite rapidly compared to the rDNA sequences and even single copy genes. Insect rDNA genes may also contain introns and/or various other middle repetitive sequences (21). In the dipterans thus far examined these genes are generally present in excess of 200 copies per genome and are arranged in a few large tandem arrays. In short, the NTS regions (and possibly introns) possess the same advantages as do mitochondrial DNA of high copy number per cell and probable divergence between species.

In order to examine the possibility that rDNA probes would be useful, Alina Mendez (an undergraduate honors student) used restriction digests of whole genomic DNA probed with the Sciara coprophila clone diagramed below:



The clone contains an 8.4kb repeat unit (shown in red) which contains both 18s and 28s genes. These genes in turn contain sequences which are highly conserved between species. Although the restriction patterns shown in fig. 2 are generally similar, the SmaI pattern shows a striking difference, thus indicating this avenue was worthy of further study. We therefore proceeded to isolate rDNA clones from A. gambiae.

A group of 13 mosquito rDNA clones were isolated from an A. gambiae library in EMBL-3 by Alina Mendez. The library (kindly provided by Nora Beszansky) was probed (42°, 50% formamide) with the 8.4kb segment of S. coprophila rDNA (22) shown above in red. After preliminary restriction analysis it was clear that several different patterns were represented, indicating that the rDNA genes of A. gambiae are probably not a homogeneous array. Insect rDNA is known to vary, particularly with respect to the presence of introns (21,23).

In order to quickly identify potentially interesting regions, two apparently different clones, AM23 and AM12, were chosen for further study. Southern analysis of EcoRI plus SalI digests probed with the Sciara rDNA revealed hybridization to a 6.64kb fragment present in both clones and a 1kb fragment (see figure 3) present only in AM12. The non-hybridizing fragments are indicated in green on the figure as dotted lines. Since by definition these fragments are non-conserved with respect to Sciara, we assumed that they might represent NTS, intron, or flanking (unique) sequences. However, the latter possibility is very unlikely. A list of the fragments with their molecular sizes is given in Table 2. In order to quickly determined whether any of the non-hybridizing fragments would reveal any useful RFLP differences, the fragments were electroeluted from preparative digests of AM12 and AM23. Due to the closeness of the fragments at 1.40 and 1.27kb, these were eluted together as a doublet as were the 0.90 and 0.80kb, both from AM23. The fragments (23TR doublet, 23BR doublet, 23BX, 12TR, and 12BX) were nick-translated and used to probe EcoRI digested genomic Southern blots. As shown in figure 4, the 12TR fragment showed hybridization in arabiensis at 5-7kb while gambiae showed a somewhat different pattern of hybridization in the 5-15kb range. Since these size differences were modest and not particularly useful for single mosquitoes we have not studied 12TR any further. In contrast, the 12BX probe revealed remarkable differences: in gambiae, a 1.3kb band hybridizes whereas arabiensis shows hybridization at 6-7.7kb, as shown in figure 5. Hybridization of 12BX to genomic EcoRI plus SalI digests shows hybridization at 0.5kb (presumably with itself) in both strains. However, 12BX hybridizes to

three additional bands in gambiae, 1.43kb, 1.3kb, and 0.9kb, which are not present in any of the arabiensis strains. Thus, because of the size differences in hybridizing bands, 12BX is probably our best candidate for use as a diagnostic probe on single mosquito blots. The 23BX pattern is identical to that seen with 12BX. When 23BX is used to probe an EcoRI plus SalI digest of AM23, it shows hybridization to itself as well as to the 23TR and 23BR doublets, but we have not yet determined whether one or both fragments of a doublet are hybridizing. Thus 23BX shares some homology with 23TR and 23BR.

With EcoRI digests the 23TR probe hybridizes strongly to 5.1kb, 3.8kb, and 2.5kb in arabiensis, but these bands are not found in gambiae as shown in figure 6. However, there are some common bands hybridizing at the higher molecular weights. Weak hybridization is seen to the 1.3kb EcoRI band, revealed by 12BX and 23BX. The SalI digests show most hybridization in gambiae is concentrated around a 1.4kb band which represents the top (SalI) band of the 23TR doublet. This fragment does not hybridize in A. arabiensis strains. At higher molecular sizes, both species show similar hybridization patterns. For EcoRI plus SalI digests most hybridization is occurring in gambiae at the 1.4 and 1.27kb bands which presumably represent the 23TR doublet, but these fragments do not hybridize in the arabiensis strains. Our interest in 23TR revolves around the possibility that its very much stronger hybridization with gambiae compared to that in arabiensis could make it useful as a species specific probe for dot blots of whole undigested single mosquitoes.

Hybridization patterns obtained from the 23BR probe are very similar but not identical to those obtained from 23TR. EcoRI digests show hybridization to 5.1kb and 3.8kb bands of A. gambiae, which are also found in the EcoRI pattern seen with 23TR. There is strong hybridization to the 1.3kb EcoRI band seen with the 12BX, 23BX, and 23TR probes. In A. arabiensis, EcoRI digests probed with 23BR show hybridization in several bands around 5.5kb. Using SalI digests we see the same general pattern found with other probes: most hybridization in A. gambiae occurs at low molecular weights while for the A. arabiensis strains it occurs at high molecular weights. Thus, the 23BR, 23TR, and 23BX probes are all potentially useful for distinguishing A. gambiae from A. arabiensis. However, we felt that 12BX (and 23BX) were most useful because of their complete lack of overlap in the areas of hybridization.

In order to further study the properties of fragment 12BX, we subcloned it into a vector "M13+ Bluescribe," purchased from Vector Labs. This vector has a multiple cloning site flanked by T4 and T7 promoters so that single stranded RNA probes (sense and antisense) can be generated. We could also, if necessary, have the vector behave as an M13 phage and thereby sequence the inserted fragment. We have definitely confirmed that the subcloned 12BX reveals a Southern pattern identical to that of the 12BX fragment we studied by electroeluting from a digest of AM12.

(D) Partial Characterization of Fragment 12BX. In order to determine the approximate location of 12BX within clone AM12, a series of restriction digests of AM12 were made using EcoRI and SalI. These were blotted and probed separately with three fragments obtained from the Sciara rDNA clone, as well as with 12BX. The origin of the Sciara probes as well as the results obtained are shown in Table 3. It is important to note that the 28s ß probe also contains the "gap" sequence plus 270bp of the 28s α coding region. The 28s α probe contains the internal transcribed spacer, the 5.8s coding sequence and 225bp of 18s sequence. Due to these overlaps in the probes, the hybridizations were not all as clear cut

as we would like. Nonetheless, it is possible to construct a crude restriction map giving the probable location of 12BX as shown in figure 7. From this we tentatively assume that 12BX represents a portion of an intron in the 28s β gene.

A second property of 12BX is that the hybridization patterns it reveals on Southern analysis are identical to those of 23BX. The relationship of these fragments to each other and to 23BR and 23TR is currently being worked out using subclones of the EcoRI/SalI portions of these doublets. Thus far we know that 12BX is not homologous to either of the EcoRI/SalI fragments. Thus whatever similarities we see in the Southern patterns revealed by these fragments must be due to sequences found in the other portion of each doublet.

(E) Southern Analysis of Single Mosquitoes. Our goal is to determine whether single dried mosquitoes from various populations of the gambiae and arabiensis species can be reliably classified using one or more of our probes.

Our first experiments with single mosquitoes compared dried (2 weeks), ethanol preserved, and frozen individuals. Basically, we used 4 mosquitoes for each treatment and probed the blot with 12BX. After a 3 hour exposure, most of the lanes could be correctly classified, with the dried mosquitoes showing the strongest hybridization. We incorrectly assumed (on the basis of this limited trial) that the analysis of single mosquitoes would present no problems. We thus began to test the reliability of the probe by accumulating blots of hundreds of individuals from the CDC colonies. As this was being done, we were attempting to subclone 12BX and the other potentially useful fragments. However, when the blots were finally probed, we found that we had two problems. One, the DNA, although present in sufficient quantity, was not restricted. If that occurs one sees a dark smear of uncut material at relatively high molecular sizes. Two, we found that we often did not obtain a sufficient amount of DNA as judged by lanes with no hybridization.

Obviously, to some extent these problems are related. Figure 8 illustrates some of the difficulty: the photograph (figure 9) shows a reasonably acceptable DNA quantity in each lane. Given this amount of DNA, lanes 3, 4, and 5 appear not to have transferred during blotting. Lane 2 appears as if it transferred but did not cut. Only lanes 7 and 8 are scoreable as A. arabiensis.

As shown in fig. 10, we have placed various amounts of whole genomic DNA in lanes 2-7. Lanes 8-12 contain single mosquitoes, dried for 1 month before extraction. Lanes 2-7 show that the gambiae DNA (KWA) and arabiensis DNA (Kisumu) give the expected patterns. Lane 8 shows partially digested gambiae DNA which hybridizes at high molecular sizes as does arabiensis, but only faintly at about 1.5kb as we expect it to do. Thus we could reliably score only lane 10 as gambiae and 12 as arabiensis.

The problems associated with the extraction, recovery, and ability to cut DNA from single mosquitoes does not thus far appear to depend upon the length of dessication for the limited times we've tested. Although the problem of poor transfer of DNA from the gel to the filter is sporadic, we attempted to use an electroblot device marketed by IBI specifically designed for horizontal agarose gels. The apparatus was so cumbersome and time-consuming to use that we've abandoned further consideration of the blotting problem.

A second possibility could be the sensitivity of the probe, such that we would literally miss hybridization in lanes which had a relatively small amount of DNA. We tried to raise the sensitivity of our probe in two ways. First, we compared 2 x 10^7 cpm/μg of the electroeluted 12BX fragment with the same specific activity for the nick-translated whole plasmid. Although 12BX is only about 12% of the total plasmid sequences, we obtained equally good results with each probe. Thus, increasing our specific activity about ten-fold did not appear to increase the sensitivity. Second, we made a single-stranded (RNA) version of 12BX, again in an attempt to rescue some of the apparently blank, unscoreable single mosquito lanes. We found that this probe gives a lower background but the signal was weaker, so that it does not amount to any increase in sensitivity. Moreover, the utilization of single-stranded RNA is inherently less practical than double-stranded DNA probes because everything (including solutions and gel boxes) must be made RNAse-free.

The DNA extraction protocol appears to be the major culprit both in terms of recovery and the ability to restrict the DNA. We have therefore used alternative protocols for DNA extraction, but as yet we do not know if they will prove to be more effective.

The analysis of single dried abdomens has been one of our long-term goals. Due to our sporadic success with the whole mosquito, we've thus far only done one experiment with abdomens. Figure 11 shows that analysis: 11 single dried abdomens from A. gambiae were run on the gel, and 7 of them are clearly scored as A. gambiae. The other lanes would not be scored at all due to either lack of DNA or DNA that is obviously not restricted. Thus, we look forward to continued success in this regard.

In summary, what we've demonstrated thus far is as follows:

1. The probe 12BX (when nick-translated as part of the M13 Bluescribe vector) is perfectly capable of giving us an easily recognized, distinctive hybridization pattern difference using whole genomic DNA.

2. Individual dried mosquitoes contain a sufficient number of copies of the rDNA sequences to allow clear differentiation between the species. This probably applies as well to single dried abdomens.

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Table 1. List of mosquito strains available from the CDC.

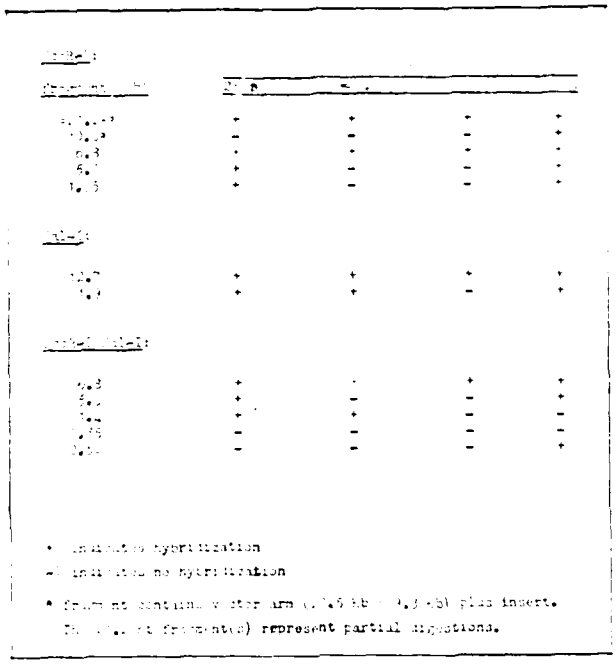
Designation	Origin	Species
GMAL	Sudan	A. arabiensis
SENNAR	Sudan	A. "
KISUMU	Kenya	A. "
G-3	The Gambia	A. gambiae
KWA	Kenya	A. "
BAD	Nigeria	A. "
MU	Tanzania	A. "
ZAN	Zanzibar	A. "
BREF	The Gambia	A. "

Table 2. Restriction fragments from A. gambiae clones AM12 and AM23 showing no homology to the Sciara heterologous probe.

fragment (Kb)	fragment origin	designation
clone 23:		
1.40	Sal-I	23TR
1.27	EcoR-I/Sal-I	23TR
0.90	EcoR-I/Sal-I	23BR
0.80	Sal-I	23BR
0.50	EcoR-I/Sal-I	23BX
clone 12:		
5.3	EcoR-I	12TR
0.90	EcoR-I/Sal-I	12TX
0.50	EcoR-I/Sal-I	12BX

* Separate EcoR-I and Sal-I digests were performed in order to determine the fragments' ends

Table 3. Hybridization profiles of EcoRI, SalI, and EcoRI/SalI restriction fragments of clone AM12 to different probes whose origin is shown below.



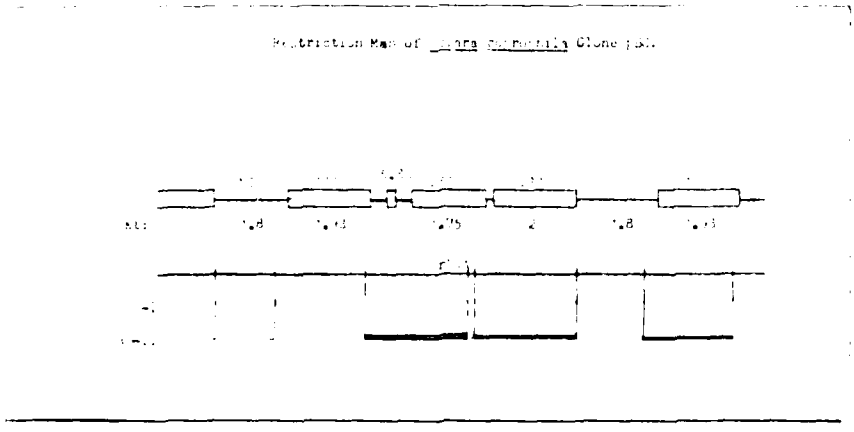


Figure 1. Autoradiogram from Southern analysis of whole genomic DNA (5 µg/lane). The probe was nick-translated to a specific activity of 4.5×10^7 cpm/µg. The electrophoresis and blotting were done according to standard protocols (24). The prehybridization and hybridization (20 hrs.) solutions contain 50% formamide, 6 x SSC, 0.5% SDS, 5 x Denhardts, 10 µg/ml salmon sperm DNA. The blots are washed at 42° for 2 hrs. This blot was exposed for 20 hrs. at -80°C. The position of the lambda EcoRI standards is given on the right.

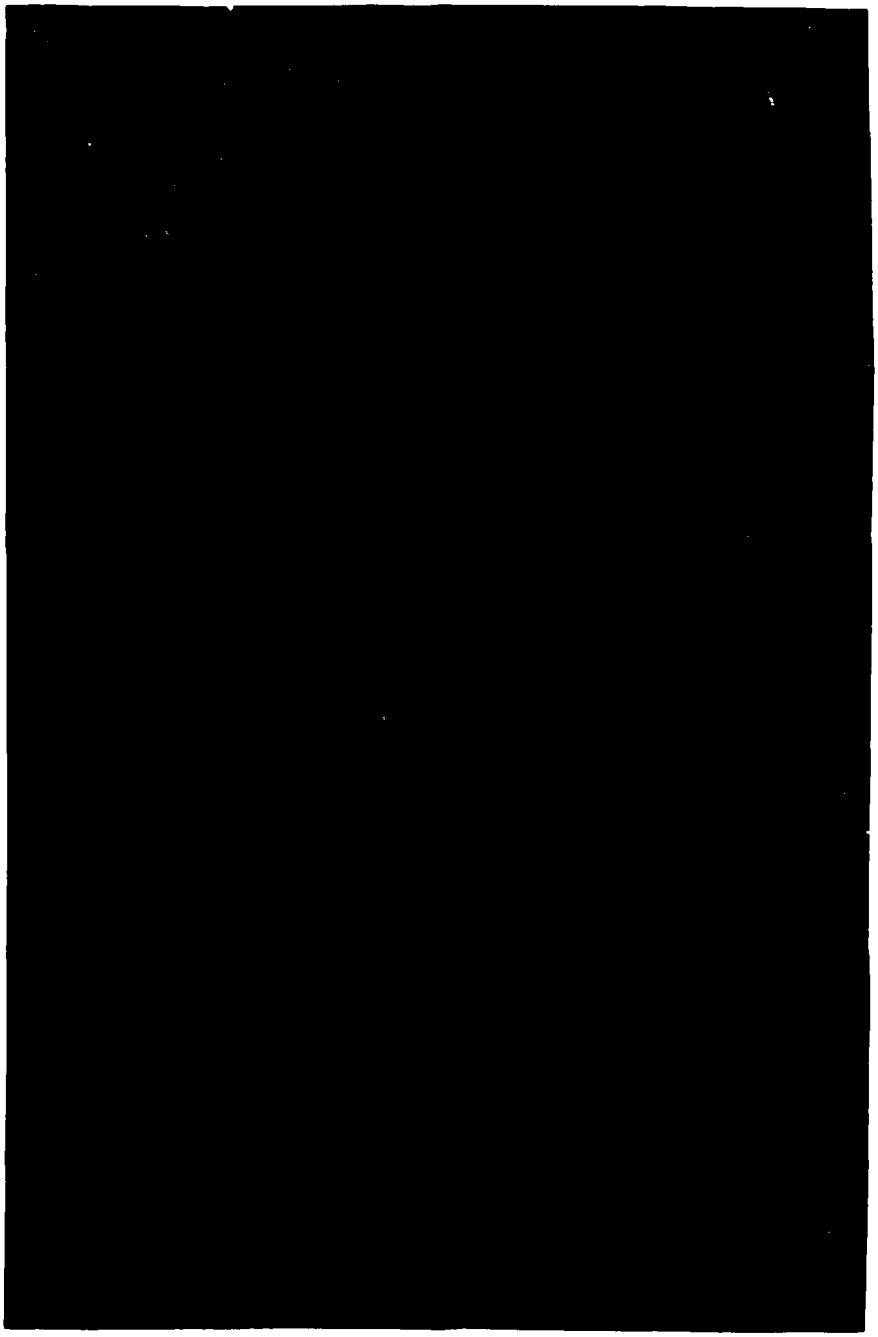


Figure 2. Hybridization of S. coprophila EcoRI repeat unit to various restriction digests of A. gambiae, strain G3 (indicated as g) and A. arabiensis, strain Kisumu (indicated as a). Left to right, lanes 1, 2 are EcoRI digests; 3, 4 are HindIII; 5, 6 are SalI; 7, 8 are SmaI; 9, 10 are HaeIII. The blot was treated as described in fig. 1, and exposed for 72 hrs. Each lane contains 5 µg DNAs. The probe was 1.4×10^7 cpm/µg. The position of the lambda R1 standards is shown on the left.

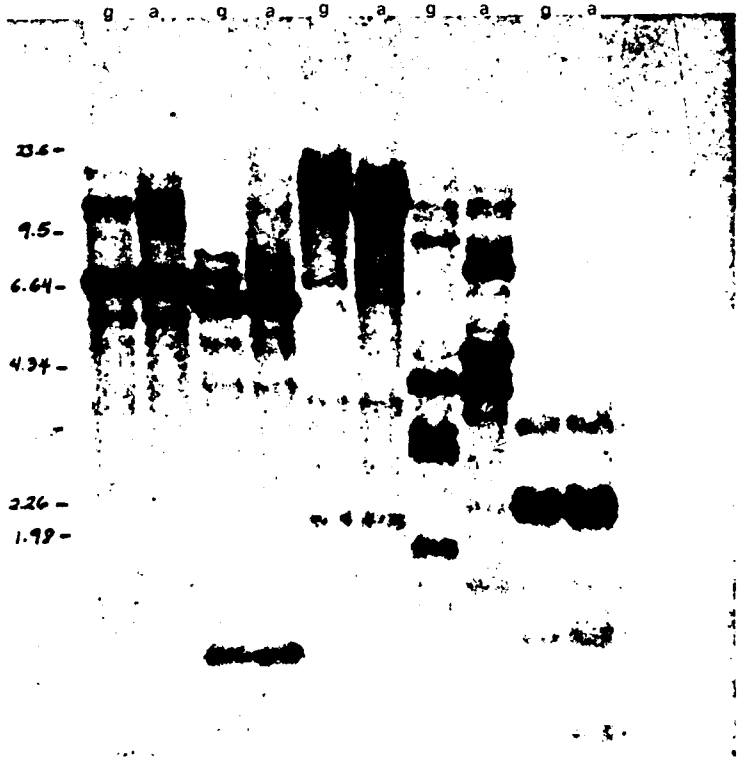


Figure 3. Hybridization patterns of clones AM23 (a, at the right) and AM12 (b, at the left) to the Sciara rDNA repeat unit. The relative locations of the non-hybridizing fragments are shown as horizontal green dotted lines. The lambda R1 standard positions for each gel are shown on the extreme right and left of the figure. Specific activity of the probe is 3.6×10^7 cpm/µg. The blot was exposed for 2.5 hrs. Note that 12TR actually does hybridize faintly to the probe. All other manipulations were described in fig. 1.

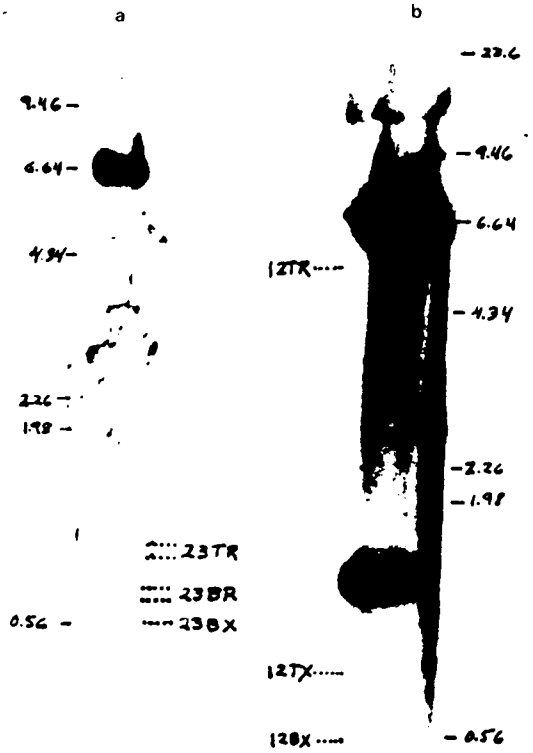


Figure 4. Hybridization of fragment 12TR to EcoRI digests of A. gambiae (strains G-3 and BAD) and A. arabiensis (strains Gmal and Sennar). The probe was 1.1 x 10^7 cpm per µg; the blot was exposed for 1 hr. All other manipulations were as described in the legend to fig. 1.

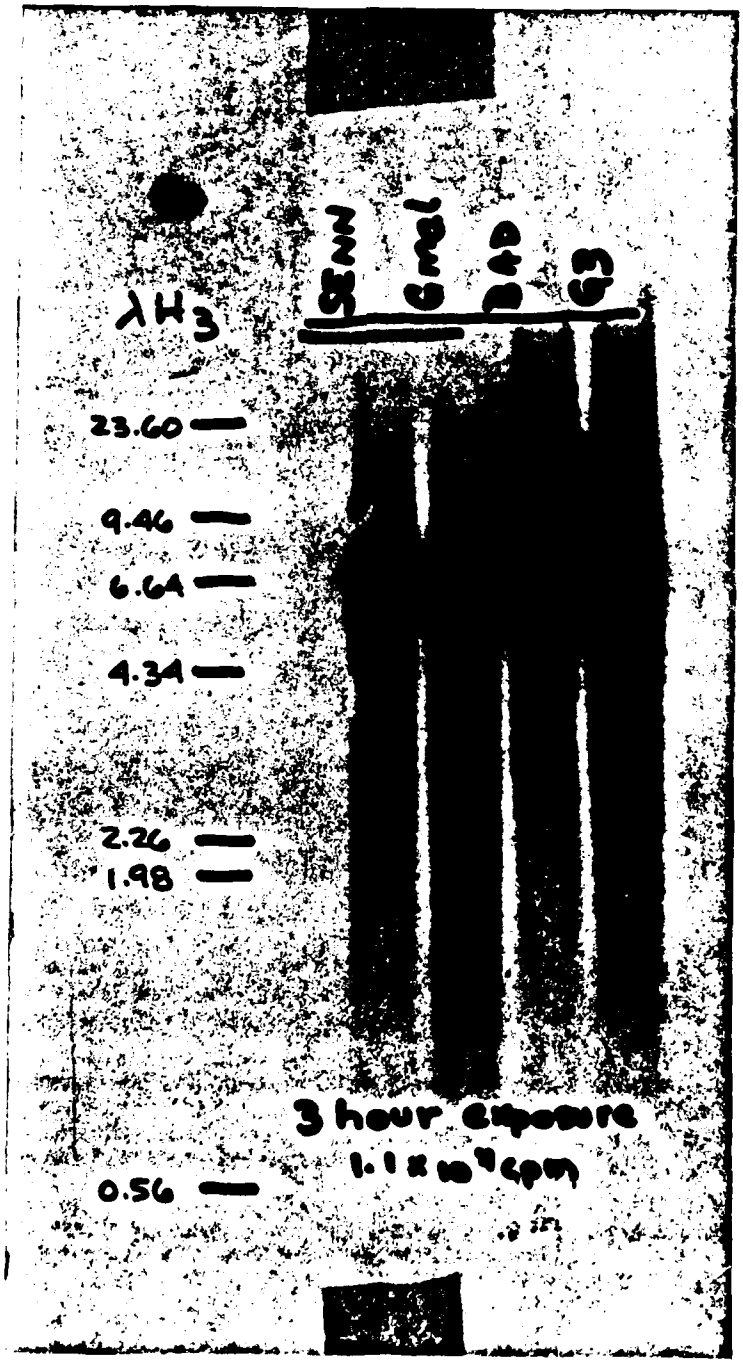


Figure 5. Hybridization of probe 12BX to EcoRI digests of A. gambiae, strains G-3 and BAD (B) and A. arabiensis, strains Gmal (G) and Sennar (S). The specific activity of the probe was 3.6×10^7 cpm/µg and the blot was exposed for 3 hrs. All other manipulations are as described in the legend to fig. 1.

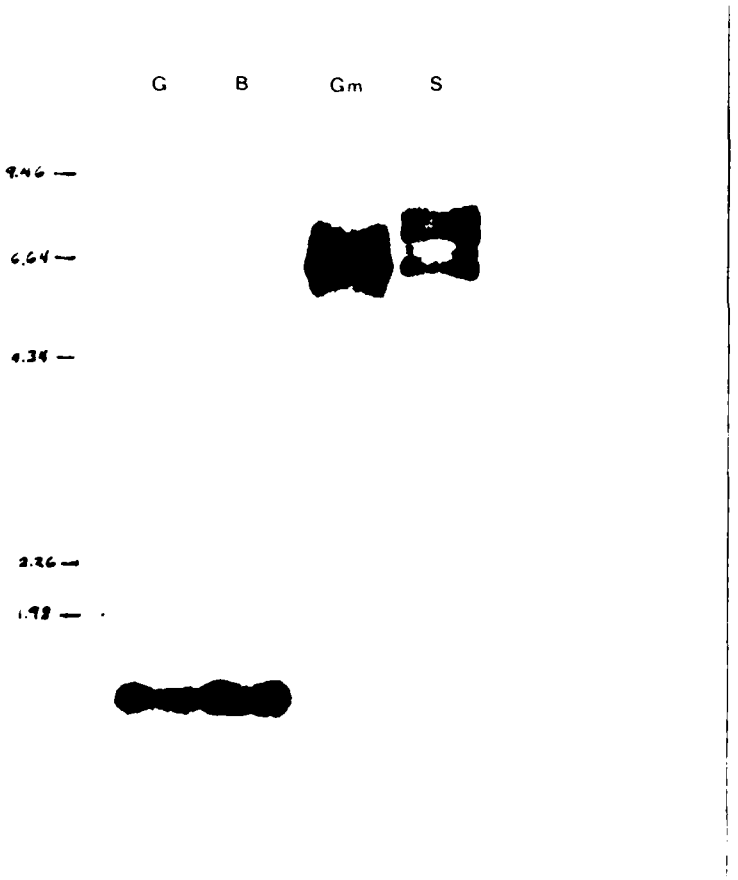


Figure 6. Hybridization of probe 23TR to EcoRI restriction digests of A. gambiae, strains G-3 (G) and BAD (B), and to A. arabiensis strains Kisumu (K), Sennar (S), and Gmal (Gm). The blot in panel 1 was exposed for 20 hrs., the blot in panel 2 was exposed for 3 hrs. The specific activity of the probe was 2.8×10^7 cpm/μg. All other manipulations were performed as described in the legend to fig. 1. Here we note that the hybridization to the arabiensis strains is much weaker than to gambiae.

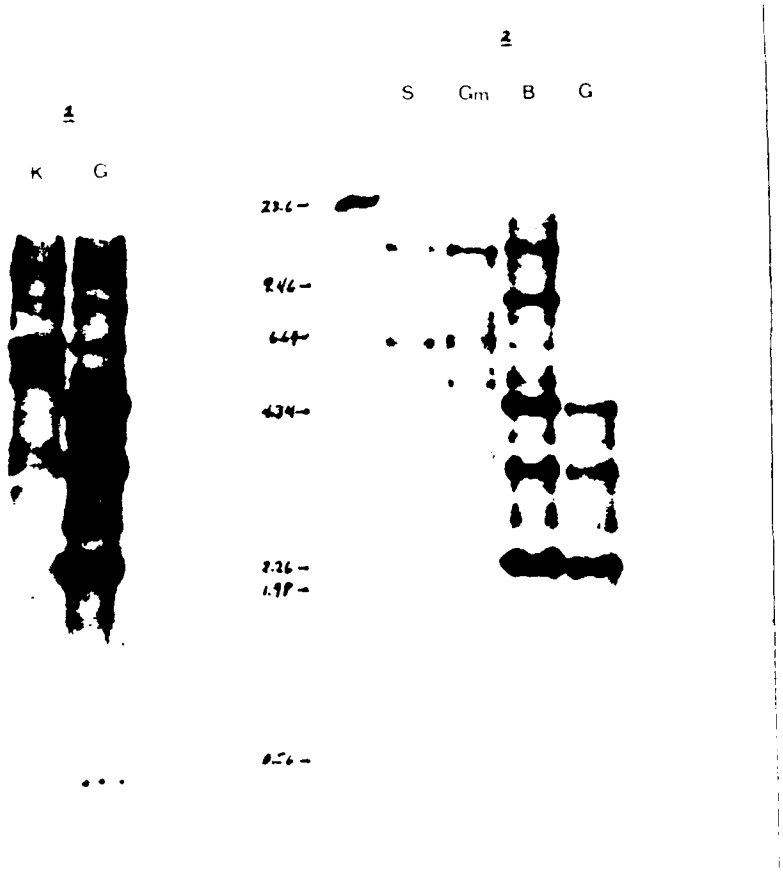
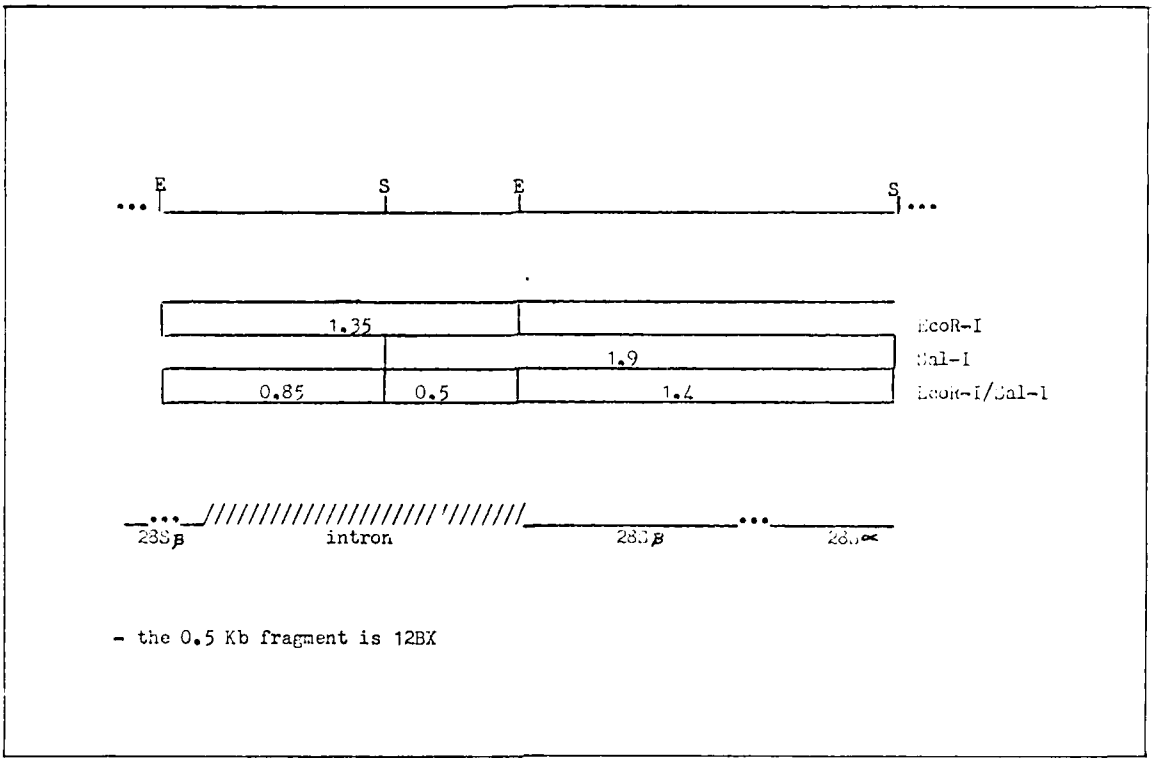
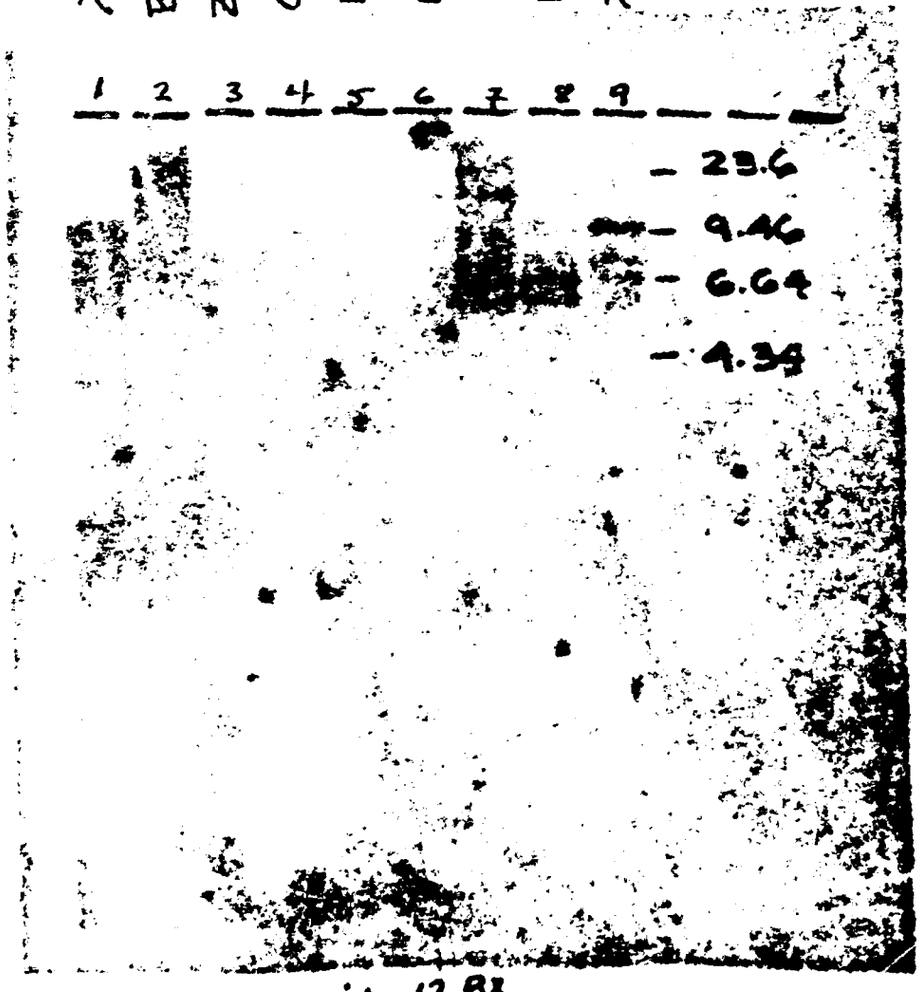


Figure 7. Tentative restriction map of a 12BX containing region of clone AM12.



- the 0.5 Kb fragment is 12BX

single mseq.



5-6 whole plasmid 12 Bx
1.8 x 10⁷ cpm (6-5-86)

3day exp
dev 6/16/86

Figure 9. Photograph of ethidium fluorescence. The strains BAD, Zan, and G-3 are A. gambiae. The Bref is A. melas; Gmal and Kisu are A. arabiensis. The live mosquitoes were placed briefly at -20°C to kill them and were then placed in separate tubes in an airtight container containing dessicant. After drying for three wks., the mosquitoes were homogenized and extracted directly in those tubes.

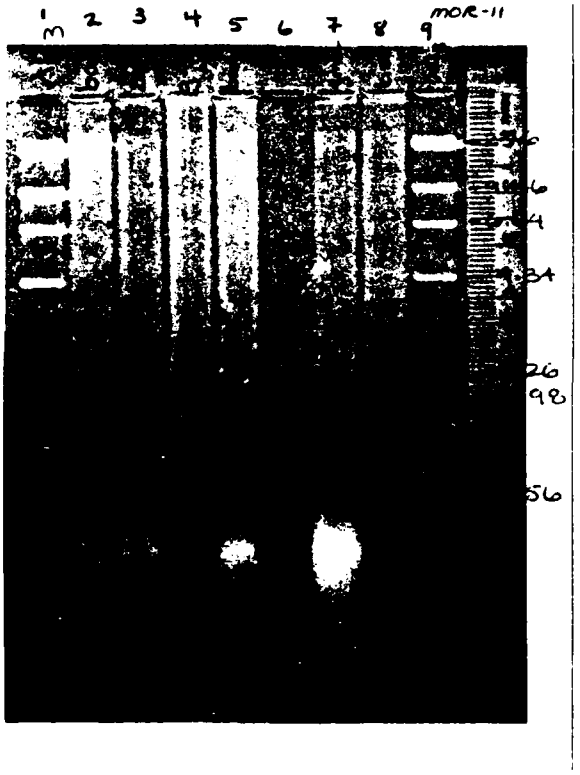


Figure 10. Southern analysis of A. gambiae and A. arabiensis. Lanes 2-7 contain whole genomic DNA placed there in order to confirm the expected RFLP pattern, as well as to compare the intensity of ethidium bormide fluorescence with that seen in the single mosquito lanes. The KWA is A. gambiae and Kisumu is A. arabiensis.

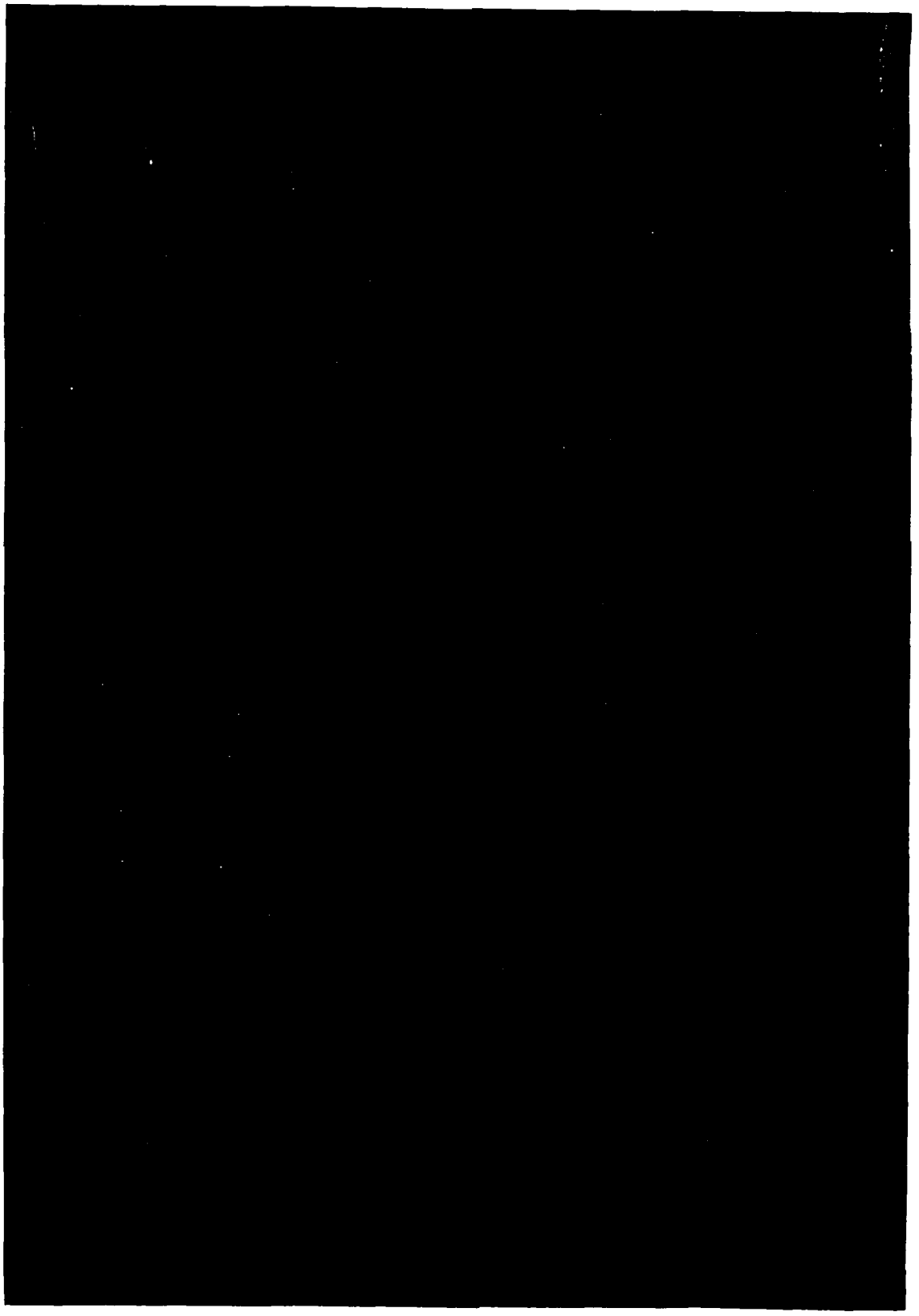
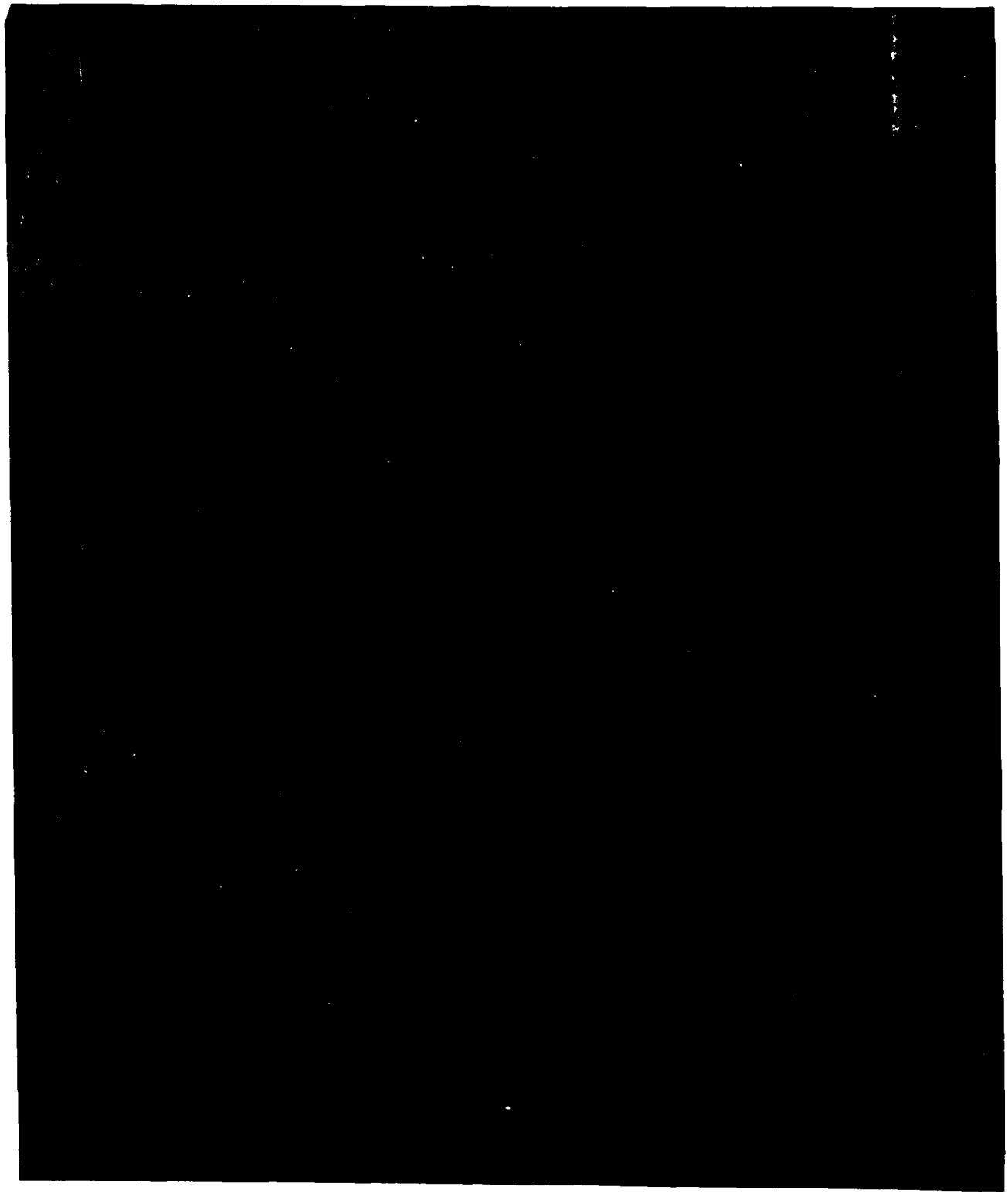


Figure 11. Southern analysis of single dried abdomens from the Zanzibar strain of A. gambiae. Lanes 4, 6, and 9 apparently have insufficient DNA to allow hybridization signal. Lane 3 has sufficient but apparently non-cut DNA. It is possible that since lanes 6, 8, and 11 do show ethidium bromide fluorescence, sufficient DNA is present for hybridization, but these individuals simply lack homology to 12BX. We are currently testing this idea by hybridizing this blot to 28s sequences from C. erythrocephala.



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